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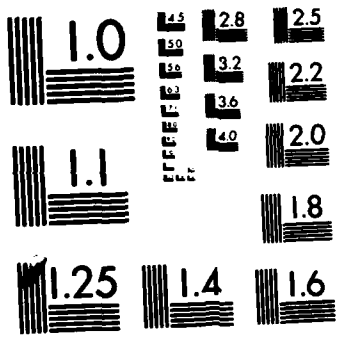

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Although the synthetic adjuvants muramyl di-peptide (MDP), polyadenylic-polyuridylic acid complexes (poly A·poly U) as well as bacterial lipopolysaccharides enhance the antibody response when given with antigen, each also can suppress markedly this response when given before antigen. The long term objective of this study is to determine whether each adjuvant suppresses the immune response by a common or different cell and molecular signal. Our findings during the first year were as follows:

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(1) Optimal conditions were determined for suppression of PFC formation by MDP and poly A·poly U in Balb/c mice. Suppression was induced by a single dose of 300 μg MDP (iv or ip) or poly A·poly U (ip) when injected 1-2 days prior to injection of antigen (SRBC).

(2) Suppression of PFC by MDP could be transferred to syngeneic recipients by whole spleen cells, by adherent cells alone or by non-adherent cells (at a somewhat lesser order of magnitude) when removed from mice 1 day after MDP. Transfer of T cells or B cells alone (purified by panning) resulted in enhancement of PFC formation.

(3) Contrariwise, suppression of PFC could not be transferred to syngeneic mice by whole spleen cells removed from Balb/c mice 1 day following pretreatment with poly A·poly U.

(4) On the other hand, serum removed from mice 90 minutes after receiving poly A·poly U was found capable of suppressing cell mediated immunity, as measured by the mixed leucocyte reaction (MLR). This effect appeared to wane by 18 hr.

Characterization of Non-Specific Immunosuppression Induced by Muramyl Di-peptides

Early experiments focused on determination of the parameters of the suppression induced by muramyl di-peptides (MDP) when the latter was injected i.v. into 8-16 week old female Balb mice. The minimum dosage was determined in several experiments utilizing dose response curves. The MDP was administered i.v. in varying quantities on Day -2, antigen (sheep red blood cells) was administered on Day 0 and the spleens removed from the mice on Day 4 to determine antibody forming cells (PFC). The minimum dose required to suppress the antibody response by 50% was found to be 300 μg. Using this dosage, we studied the kinetics of MDP suppression and found that suppression was induced by MDP when injected either 1 or 2 days prior to antigen, and that a single dose was as effective as 2 consecutive doses (Table 1).

TABLE 1

Per Cent Suppression* by MDP (300 μg i.v.) Given

	Day -2	Day -1	Day -2 and -1
In Vivo	43 ± 10	61 ± 18	46 ± 22
In Vitro	30 ± 2	50 ± 4	

*Mean of 3 experiments ± S.D. (Balb mice, 2 months old)

We also investigated the capacity of MDP to induce suppression in an *in vitro* system. MDP was injected into mice on Day -2 and -1 and the spleen removed on Day 0. These MDP-treated spleen cells as well as cells from control mice receiving medium alone were cultured in a Mishell-Dutton system together with 1×10^7 SRBC added *in vitro*. The MDP-treated cells were also tested for their capacity to inhibit PFC function by 5×10^6 normal cells or 5×10^6 cells exposed to medium, Hanks balanced salt solution (HBSS). *In vivo* controls consisted of MDP treated mice and HBSS treated mice which were injected (i.v.) with 1×10^7 SRBC as antigen on Day 0 and sacrificed on Day 4. The suppression achieved *in vitro* was compable to that induced *in vivo*. Injection of MDP on day -1 appeared to be optimal for *in vitro* studies with the MDP-treated cells being suppressed 50% (± 4% at .007 significance level) and this level of suppression was transferrable to normal spleen cells. Injection on Day -2 induced a mean of 30% (± 2% at .002 significance level) suppression of MDP cells (Table 1). This suppression also was fullytransferrable to normal spleen cells.

To determine which cellular population was responsible for the suppression of the *in vitro* PFC response the spleen cells were fractionated in various ways. Adherence on plastic culture dishes was the first separation technique attempted with inconsistent results. For example, in

one experiment, it appeared that the adherent cells removed from MDP treated mice (predominantly macrophages) were the most suppressive and the nonadherent (NA) cells only slightly suppressive. However, in another experiment the MDP-treated NA cells suppressed the response by 70% whereas the MDP-treated adherent cells suppressed only 38%. Consequently, we returned to the in vivo system.

Our objective in the following experiments was to determine whether MDP-induced suppression in vivo could be transferred in vivo to normal syngeneic mice through injection of MDP-treated spleen cells or serum. As seen in Table 2, these experiments indicated that the suppression induced by MDP treatment in vivo was acting primarily via a cellular component and that the whole spleen cell population was suppressive upon in vivo transfer (i.v.) to a normal recipient.

TABLE 2

Per Cent Suppression* Induced In Vivo by Transfer of

MDP Spleen Cells	Control Spleen Cells	Fold Increase	MDP Serum	Control Serum	Fold Increase
56 ± 7	12 ± 6	4.7	41 ± 2	28 ± 1	1.5

*Mean of 2 experiments ± S.D. (Balb mice, 2 months old)

To determine which cell population was responsible for suppression of the in vivo PFC response the whole spleen cell population from MDP or HBSS treated animals was applied to BHK microexudate-coated surfaces (Ackerman and Douglas, J.I. 1978) to which macrophages would adhere. After 2 hrs, the nonadherent cells were decanted and collected and the adherent macrophages detached and collected. After washing and counting these cells, they were injected into normal recipients simultaneous with antigen. The PFC response 4 days later is recorded in Table 3, where it appears both adherent and nonadherent cells exhibited some degree of suppression.

TABLE 3

Per Cent Suppression* Induced In Vivo by Transfer of

Adherent Spleen Cells		Non-adherent Spleen Cells	
MDP	Control	MDP	Control
41 ± 5	14 ± 4	33 ± 15	3 ± 6

*Mean of 2 experiments ± S.D. (Balb mice, 2 months)

However, although the difference between the adherent and NA cells was not very large, the former appeared significantly greater at the .01 level. The HBSS treated control cells did not suppress the response from the control level, nor was there any significant difference beteen adherent and non-adherent HBSS cells when separated.

Because of the lack of a clear cut separation of activity between adherent and NA cells, in the next experiment, a different type of cell separation (panning) was carried out. The whole spleen cell population from MDP or HBSS treated animals was applied to plastic culture dishes coated with rabbit-anti-mouse immunoglobulin. After incubation, the unattached cells (predominatly T cells) were decanted and collected. Next the bound cells (B cells) were detached and recovered by flushing the entire surface of the plate with PBS/1% FCS. After centrifugation, both cell types were counted, 1 x 10^7 cells injected into normal syngeneic recipients simultaneously with antigen and the PFC response measured 4 days later. Both T cells alone and B cells alone caused enhancement of the PFC response. An in vivo control, (MDP on Day -1, antigen on Day 0) showed 30% suppression (data not shown). These results suggested that T cells and B cells might be necessary together for suppression of the PFC response to be transferred.

The next series of experiments employed procedures separating and testing whole spleen cells, adherent cells, non-adherent cells, and T and B cells from mice receiving either MDP or HBSS i.v. on Day -1. In vivo controls were included and all cell populations tested simultaneously. 1 x 10^7 treated cells were injected simultaneous with antigen on Day 0, and the recipient mice were sacrificed 4 days later to measure the PFC response. Representative results are shown in Table 4.

TABLE 4

Spleen Cell Populations Capable of Transfer of Suppression

Population	% Change	P value
Whole	↓ 47	.003
Adherent	↓ 41	.005
Non-adherent	↓ 30	.002
T	↑ 45	.007
B	↑ 23	.002

All the various cell populations injected in these transfer experiments were subjected to a mitogenicity test, to determine the effectiveness of the separation techniques. Thus, the whole population, NA and T cells responded to Con A stimulation with a 10-30 fold increase in H^3-thymidine uptake while

the responses of adherent and B cells were slight (0-5 fold increase). Similarly, the whole population, NA and B cells responded with a 5-10 fold increase to LPS stimulation, while the responses of the adherent and T cells were much less (2-3 fold increase). Slight contamination may be indicated.

The next objective was to obtain preliminary evidence as to whether or not the T cell was required in the induction of suppression. In the following experiments, Balb/c-Nu/nu were injected with MDP or HBSS on Day -1 and sacrificed as spleen donors on Day 0. The spleen cells were separated by adherence techniques into adherent and NA populations. These cell populations were then injected simultaneously with antigen into normal, thymic Balb/c mice.

As can be seen in Table 5, the results of the first experiment, the nu/nu whole cell population and nu/nu adherent cells were able to suppress the PFC response by 55-60%. The nu/nu NA (B cells) caused a slight (29%) enhancement. These results indicate that the T cell is not required for MDP to induce suppression.

TABLE 5

Per Cent Suppression Induced in Normal Balb Mice by Cells from MDP-Injected Balb Nu/Nu Mice

PER CENT SUPPRESSION TRANSFERRED WITH		
Whole Spleen	Adherent Cells	Non-adherent Cells
59 ($p < .003$)	55 ($p < .001$)	↑29 ($p > .05$)

Characterization of Non-specific Immunosuppression Induced by Polyribonucleotide Complexes

Experiments also have been initiated to characterize the immunosuppression induced by polyribonucleotide complexes when the latter are injected into mice 1-2 days before antigen (SRBC). Dose-response relationships established 150-300 µg/mouse given in a single dose as optimum levels for induction of 50% suppression of PFC. Slight variation was seen between two different lots of poly A:poly U. In addition, the intraperitoneal route appeared to be more effective than the intravenous route. Results are tabulated in Table 6.

TABLE 6

Per Cent Suppression Induced by Poly A:Poly U*

Experiment Number	Per Cent Suppression	Mean ± S.D.
1	50	
2	62	
3	41	
4	70	54 ± 14
5	35	
6	63	

*150 µg ip 1 day before ip injection of 10^8 SRBC to Balb/c mice.

However, unlike the MDP induced suppression, we have been unsuccessful to date in transferring this capability to syngeneic Balb mice with whole spleen cells (Table 7).

TABLE 7

Attempts at Spleen Cell Transfer of Poly A:Poly U Induced Suppression

Spleen Cells, Transferred*	% of Control PFC	n
10^5 PBS Control	112 ± 7	2
10^5 A:U	156 ± 22	2
10^6 PBS Control	129 ± 55	4
10^6 A:U	108 ± 47	5
10^7 PBS Control	99 ± 35	10
10^7 A:U	104 ± 44	10

*Removed 24 hr after ip injection of 150 µg poly A:poly U.

An in vitro system is also being developed wherein poly A:poly U was added to normal Balb spleen cells in culture one day before addition of antigen (SRBC), and PFC's measured 4-5 days later. The results of the first experiments are shown in Table 8.

TABLE 8

Induction of Suppression In Vitro by Poly A:Poly U

	PFC/10^6 spleen cells		
Dose of Poly A:Poly U	Exp. 1	Exp. 2	Exp. 3
	103 ± 1	162 ± 52	105 ± 30
0.05 µg	84 ± 9	188 ± 62	33 ± 25
0.5 µg	36 ± 8	144 ± 26	46 ± 24
5.0 µg	29 ± 10	77 ± 77	83 ± 70

To determine whether the non-specific suppression induced by poly A:Poly U might be mediated through serum factors, mice were bled from the axillary

fossa at 1½ and 18-24 hr after intravenous injection of poly A:poly U and the serum tested for capacity to inhibit the PFC response. Variable results were observed in six experiments, and the reasons for these variations are under study.

On the other hand, an interesting suppressive effect of poly A:poly U induced serum on cell mediated immunity (as measured by the mixed leukocyte reaction, MLR) was observed. Thus, 0.05 ml of serum collected 90 min after iv injection of 600 µg poly A:poly U when added at the initiation of the MLR suppressed the response consistently, whereas this effect appeared to wane when serum was collected 18 hr after poly A:poly U (Table 9).

TABLE 9

Inhibition of MLR by Poly A:Poly U Induced Serum

Time	Injected with	Experiment No. 1 CPM	1 % Suppression	2 CPM	2 % Suppression	3 CPM	3 % Suppression	4 CPM	4 % Suppression
1½ hr	Media	15,100 ± 900	100+	17,380 ± 1,196	39	17,886 ± 1,006	34	3,649 ± 757	78
	poly A:poly U	-1,200 ± 800		10,612 ± 1,974		11,771 ± 799		812 ± 596	
18 hr	Media	8,800 ± 1,500	58	41,720 ± 1,095	55	14,761 ± 307	0	4,907 ± 571	17
	poly A:poly U	3,700 ± 900		18,593 ± 2,309		18,252 ± 779	(+ 24)	4,075 ± 1,005	

Average % Suppression = 1½ hr (63%); 18 hr (32%).

Prostaglandins appeared not to be implicated as the mediator in the serum induced inhibition, since in three experiments 300 µg indomethacin given ip to mice one hour prior to iv injection of poly A:poly U did not affect the suppression.

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